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13. ABSTRACT (Maximum 200 Words)

Successful tumor growth requires angiogenesis, or the sprouting of new blood vessels from existing ones, to supply tumor cells with essential nutrients and oxygen. In addition, cancer cells must also establish a productive interaction with their neighboring stroma, which produces a matrix environment conducive to tumor growth and may also help shield tumor cells against systemic immune surveillance. A novel angiogenic factor essential for vascular development, Cyr61, has been recently identified as a marker for invasive breast carcinomas. We have found that the receptor for Cyr61 in breast cancer cells is integrin alpha 6 beta 1. Cyr61 also inhibits the proliferation of normal breast epithelial cells and MCF7 carcinoma cells, in part through enhancing apoptotic pathways. A new hypothesis on the role of Cyr61 in breast cancer cell growth is currently being tested.

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Introduction

Successful tumor growth requires angiogenesis, or the sprouting of new blood vessels from existing ones, to supply tumor cells with essential nutrients and oxygen. Thus, the growth of tumors beyond ~1 mm in diameter is absolutely dependent on neovascularization. Inhibition of tumor angiogenesis can dramatically restrict tumor growth, and remarkable efficacy of anti-angiogenic cancer therapy has been demonstrated in a number of animal studies. In addition, cancer cells must also establish a productive interaction with their neighboring stroma, which produces a matrix environment conducive to tumor growth and may also help shield tumor cells against systemic immune surveillance. Stromal fibroblasts, rather than the tumor cells themselves, are often the primary sources of proteolytic enzymes necessary for tumor invasion and metastasis. Thus, the ability of cancer cells to induce angiogenesis and to interact with stroma are two important processes in tumor development. Identification of factors that can play roles in both processes is therefore particularly exciting. A novel angiogenic factor essential for vascular development, Cyr61 (Babic et al., 1998; Mo et al., 2002), has been recently identified as a marker for invasive breast carcinomas (Menendez et al., 2003). In this study, we examine the role Cyr61 plays in the interaction between tumor cells and stromal fibroblasts in tumor growth.

BODY

In last year's report, we have investigated the biological activities of Cyr61 on mammary epithelial and adenocarcinoma cells. We showed that Cyr61 interacts with these cells through integrin $\alpha_6\beta_1$, a cell surface receptor for Cyr61 (Chen et al., 2000). We have also characterized a number of cellular responses to Cyr61 in breast epithelial and cancer cells, including effects of Cyr61 on estrogen dependence, DNA synthesis, and synergism with EGF and TGF-β. Surprisingly, we found that MCF7 cells transfected with a Cyr61 expressing vector resulted in reduced tumorigenicity. This finding is perplexing because Cyr61 is known to be an angiogenic inducer, and enhances tumor growth in some instances. Expression of Cyr61 also enhances tumor growth in stable transfected clones of MCF7 cells (Menendez et al., 2003). However, it is important to note that in our experiments, we have used a pool of transfected cancer cells expressing Cyr61 rather than highly selected stable clonal cell lines. These results suggest that selected, transfected MCF7 cells expressing Cyr61 behave differently from tranisently transfected MCF7 cells populations.

To elucidate the effect of Cyr61, we have further characterized the cellular responses to Cyr61 in beast epithelial and cancer cells. These studies led to the discovery that Cyr61 can induce apoptosis in stromal fibroblasts, and the proposal of a new model for Cyr61 function in cancer.

1. Cyr61 reduced cell proliferation but not DNA synthesis.

Our previous studies indicated that Cyr61 synergized with TGF-β to inhibit DNA synthesis in normal breast epithelial cells, but not in breast cancer cells. To examine this observation further, we quantified cell proliferation by counting cell numbers. Both the normal mammary epithelial cell line MCF10A and the breast cancer cell lines MCF7 are growth inhibited by the presence of Cyr61 (Fig. 1). Treatment of cells with Cyr61 reduced the cell number of both cell lines significantly, yielding about only ~30% of the number of cells compared to the untreated controls.

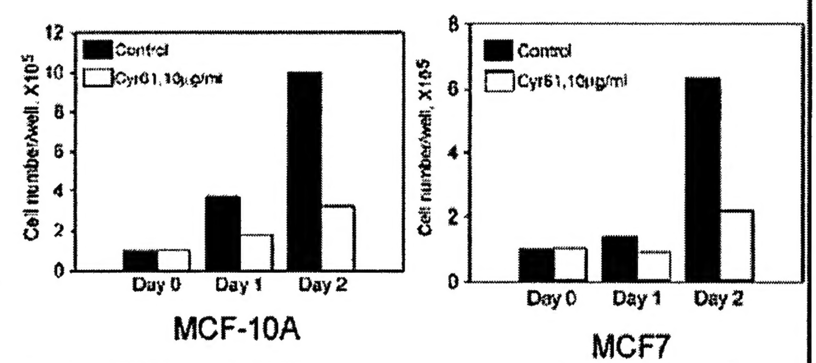
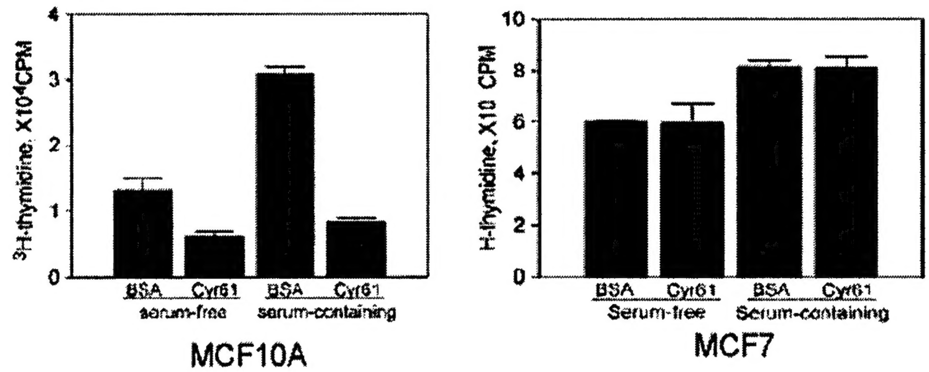


Fig.1 . MCF10A and MCF7 cells were plated on 6-well plates at 1 x 10^5 cells per well and cultured in normal growth medium. Cell numbers were counted 1 or 2 days after plating.

To study the effect of Cyr61 on cell cycle progression, serum-starved MCF10A or MCF7 cells were treated with Cyr61. DNA synthesis in MCF10A cells was inhibited in both serum-free or serum containing medium, but not in MCF7 cells. Therefore, inhibition of DNA synthesis may explain the reduction in cell numbers in MCF10A cells, but not in MCF7 cells. Thus, MCF7 cells are able to enter S phase in the presence of Cyr61 similar to untreated controls, suggesting that reduction in cell number may be

due to hening of cell cycle or age at later s of the cycle.



lengt the (G1) block stage cell

Fig.2 . Serum starved cells were treated with 10 μg/ml purified Cyr61 either in serum-free or 10% serum for 24 hrs. ^{3}H-thymidine incorporation was then determined.

2. Cyr61 induces apoptosis in MCF10A cells.

Since reduction of DNA synthesis may reflect either a cell cycle block or an increased in cell death, we examined the possibility that MCF10A cells might be undergoing apoptosis. We found that treatment of MCF10A cells by Cyr61 resulted in significant cell death as reflected by TUNEL assay, whereas MCF7 cells are not susceptible to Cyr61-induced cell death (Fig. 3). Thus, these results point to a previously unknown apoptotic activity of Cyr61. Since Cyr61 is known to confer protection from apoptosis in human umbilical vein endothelial cells (HUVECs; Leu et al., 2002), it is surprising to find that Cyr61 is actually causing apoptosis. However, this observation provides a possible explanation for the inhibition of tumor growth by Cyr61 in MCF7 cells. We hypothesized that Cyr61, secreted by the tumor cells, may be able to induce apoptosis in stromal fibroblasts, thereby reducing tumorigenicity by compromising productive tumor-stromal interactions.

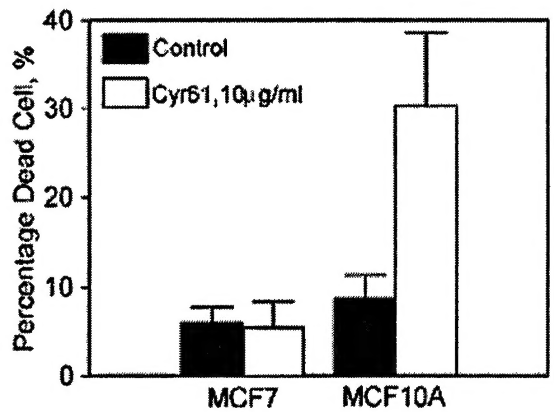


Fig. 3. Serum-starved MCF7 or MCF10A cells were treated with Cyr61 in serum-free media for 24 hrs. Apoptotic cells were assayed by TUNEL.

3. Cyr61 induces apoptosis in stromal fibroblasts.

To test this hypothesis, we examined the ability of Cyr61 to induce apoptotic cell death in fibroblasts. Also compared the effects of Cyr61 on HUVECs, since they were previously shown to be protected by Cyr61. As shown in Fig. 4, we treated HUVECs, primary human fibroblasts (1070SK), and Rat1a fibroblasts similarly with Cyr61 under serum-free conditions for 24 hrs. As we observed previously, Cyr61 protected HUVECs from apoptosis, reducing the number of apoptotic cells from ~27% to 3%. In fibroblasts, however, Cyr61 had an opposite effect, and induced cell death. In primary fibroblasts, apoptotic cells increased from ~1% to 10%, and in Rat1a cells, from ~14% to 50% (Fig. 4).

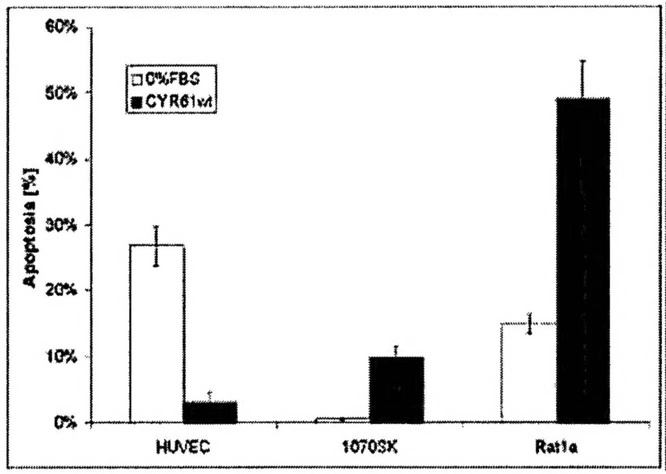


Fig. 4. HUVECs, 1070SK, and Rat1a cells were similarly treated with 10 mg/ml Cyr61 under serum-free conditions. Apoptosis was measured by TUNEL assay.

Treatment of 1064SK primary human fibroblasts with Cyr61 for 24 hrs under serum-free conditions leads to apoptosis in ~10% of the cells (Fig. 4). Since primary fibroblasts are known to be relatively resistant to apoptotic signals, we switched to a fibroblast cell line that is more susceptible to apoptosis, Rat1a cells. In these cells, a much higher percentage of cells undergo apoptosis upon Cyr61 treatment, and thus we use this cell lines to characterize Cyr61 effects on apoptosis further.

Thus, Cyr61 performs different functions in different cell types with respect to cell survival. In endothelial cells it promotes survival under apoptotic conditions, but in fibroblasts it induces apoptosis.

4. Hypothesis: Role of Cyr61 in breast cancer

A paradox emerged with the findings that expression of Cyr61 in stable cell lines derived from MCF7 cells promotes tumor growth (Menendez et al., 2003), but in transfected cell populations suppresses tumor growth (our results). In addition, Cyr61 acts as a tumor suppressor in non-small cell lung cancer (Tong et al., 2001). Based on the results obtained so far, we have gained a new perspective on Cyr61 functions and developed a new hypothesis regarding its function in cancer.

Cyr61 is an angiogenic molecules, and promotes endothelial cell growth and survival. Its role in wound healing and development may require an apoptotic function in other cell types such as fibroblasts or normal breast epithelial cells (Figs. 3,4). Some cancer cells may have developed resistance to the apoptotic effects of Cyr61, and in these cases, expression of Cyr61 can promote tumor growth given its angiogenic activity. However, if the apoptotic function of Cyr61 on either the cancer cells or stromal fibroblasts out-weigh the angiogenic effect, then Cyr61 can suppress tumor growth. Thus, depending on the specific response of the tumor cells to Cyr61 or to stroma, expression of Cyr61 may be either beneficial or detrimental to tumor growth.

5. Mechanism of Cyr61-induced apoptosis.

To investigate the mechanism of Cyr61-induced apoptosis, we first examined its caspase dependence. As shown in Fig. 5A, the pan-caspase inhibitor Z-VAD completely blocked Cyr61-induced cell death, as did the caspase 3 inhibitor Z-DVED and the caspase 9 inhibitor Z-LEHD. However, the caspase 8 inhibitor Z-IETD had no effect. Therefore, Cyr61-induced apoptosis is dependent on caspase 9 and caspase 3, but not on caspase 8. These results indicate the activation of an intrinsic mitochondrial pathway of apoptosis. To investigate this possibility further, we have used Rat-1a cells lines that have been transfected with expression vectors of the anti-apoptotic proteins Bcl-2 or Bcl-X_L (Fig. 5B). Expression of either Bcl-2 or Bcl-X_L were able to abolish Cyr61-induced apoptosis, suggesting that Cyr61 causes mitochondrial release of cytochrome C to activate caspase 9 and consequently the effector caspase 3 (Newmeyer, DD and Ferguson-Miller). To test this hypothesis, we examined cytochrome C release by immunofluorescence (Fig. 5C). Control cells showed localized staining characteristic of intact mitochondria, whereas treated cells showed blurred staining indicating that cytochrome C was released to the cytoplasm through compromised mitochondrial membranes. Futhermore, Bax was also activated and localized in Cyr61-treated cells. Together, these data show that Cyr61 induces cytochrome C release from the mitochondria, resulting in cell death.

6. Receptors mediating Cyr61-induced apoptosis.

Since Cyr61 is a ligand of integrins and mediates its activities through integrin receptors, it is likely that its apoptotic actions are also mediated through integrins. In particular, Cyr61 acts on fibroblasts through integrin $\alpha_6\beta_1$. We therefore investigated whether the apoptotic effects of Cyr61 can be blocked by anti-integrin α_6 monoclonal antibody GoH3. As shown in Fig. 5D, GoH3 blocked Cyr61-induced apoptosis, but normal IgG had no effect. These results indicate that Cyr61 induces apoptosis through its receptor in fibroblasts, integrin $\alpha_6\beta_1$.

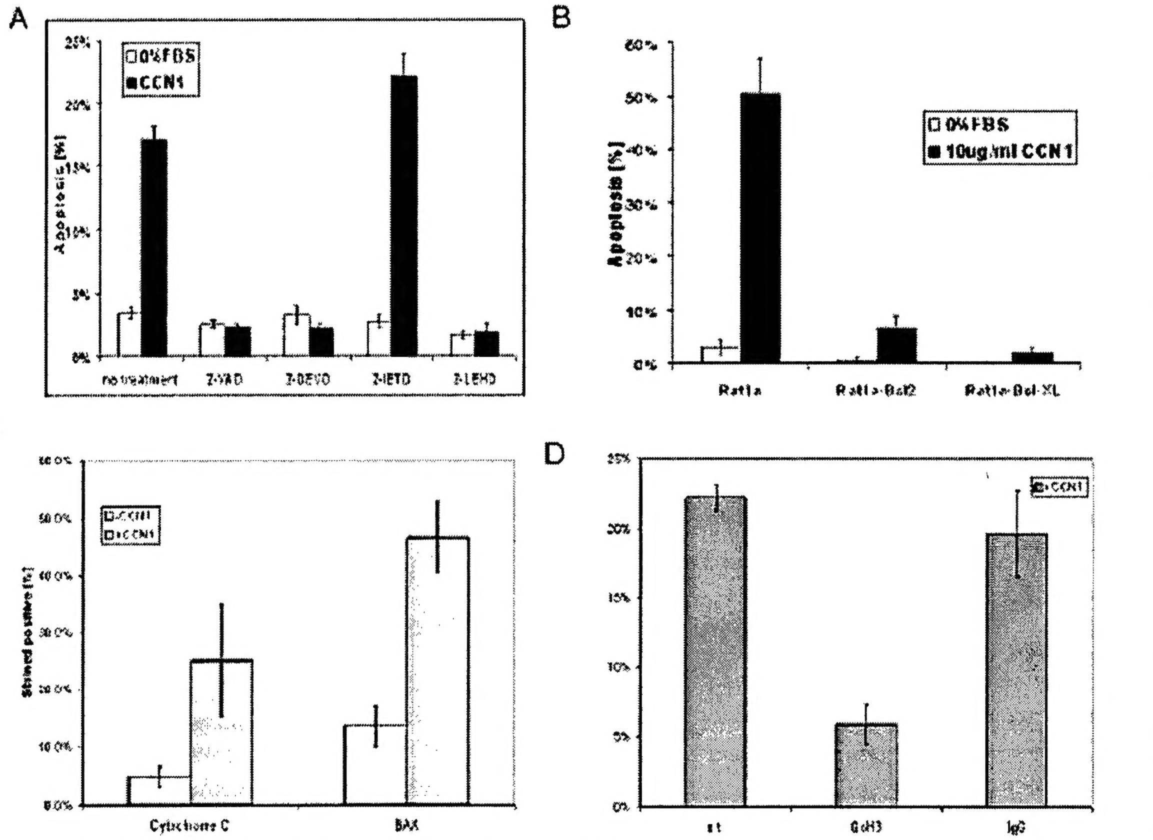


Fig. 5. Mechanism of Cyr61-induced apoptosis. A. Rat1a cells were treated with indicated caspase inhibitors with or without Cyr61 and assayed for apoptosis. B. Rat1a parental cells or cells stably expressing Bcl-2 or Bcl-XL were treated with Cyr61 and assayed for apoptosis. C. Cells were fixed, permeabilized and stained with cytochrome C or anti-Bax antibodies and rhodamine conjugated anti-mouse antibodies. D. Cell death was assayed in Rat1a cells treated with Cyr61 alone (nt) or with GoH3, or IgG.

Key Research Accomplishments:

- Demonstrated that Cyr61 inhibits proliferation of MCF10A and MCF7 cells
- Showed that Cyr61 inhibits DNA synthesis in MCF10A but not MCF7 cells
- Showeded that Cyr61 induces apoptosis in MCF10A but not MCF7 cells
- Demonstrated that Cyr61 induces apoptosis in stromal fibroblasts
- Showed that Cyr61 induces apoptosis through activation of the intrinsic mitochondrial pathway
- Showed that Cyr61 induces apoptosis through a novel pathway mediated through integrin $\alpha_6\beta_1$.

With respect to the Statement of Work, our accomplishments are:

Task 1 (1-18 months). *To determine the biological activities of Cyr61 on breast epithelial cells.*

We have accomplished this goal.

Task 2 (1-36 months). *To assess the role of stromal Cyr61 in breast tumor development*

Our experiments along this aim led to the unexpected finding that MCF7 cells overexpressing Cyr61 have reduced tumorigenicity. This observation is based on a transfected pool of MCF7 cells, rather than highly selected stable cell lines that express a high level of Cyr61.
These findings required us to investigate the reasons for Cyr61 to suppress tumor growth, leading to the surprising discovery that Cyr61 can induce apoptosis in stromal fibroblasts. These findings open up new and important questions in Cyr61 mediated cell signaling and tumor growth.

Task 3 (18-36 months). *To assess the effectiveness of blocking Cyr61 activity as a potential breast cancer therapy.*

This aim has not yet been initiated. These studies will be contingent upon results from current studies regarding the role of CCN1 in stromal cell apoptosis.

REPORTABLE OUTCOMES

Discoveries: Cyr61 induces apoptosis in primary human fibroblasts and in Rat1a fibroblasts. Apoptosis is mediated through mitochondrial release of cytochrome C and is dependent on Cyr61 interaction with integrins.

Training: These experiments have further the training of a postdoctoral fellow, Dr. Chih-Chiun Chen, whose expertise in molecular biology is now complemented by experience is tumor biology and signal transduction.

Publication: None

CONCLUSIONS

In the past year, our studies on Cyr61 action on breast cancer cells led toe the surprising discovery that Cyr61 can induce apoptotic death in fibroblasts. In addition, this apoptotic action is novel and proceeds through ligand interaction with integrins. Understanding the mechanism of this apoptotic action will lead to new insights into survival control mechanisms and cell signaling pathways.

In the context of breast cancer, our findings provides the basis for a new model for Cyr61 action that explains a number of seemingly contradictory observations. Cyr61 can be either beneficial or detrimental to tumor growth, dependent on whether the tumor cells is susceptible to Cyr61-induced apoptosis, and the degree to which the tumor cells dependent on the neighboring stromal fibroblasts. If the tumor cells and/or neighboring stromal develop resistence to Cyr61-induced cell death, the expression of Cyr61 is beneficial since it is angiogenic. The mechanisms of Cyr61 induced apoptosis and cancer cell resistance is currently under investigation.

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Appendices

None